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An electron-microscopical analysis of capture and initial stages of penetration of nematodes by *Arthrobotrys oligospora*

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A detailed analysis was made of the capture and subsequent penetration of nematodes by the nematophagous fungus *Arthrobotrys oligospora* using different electron-microscopical techniques. Capture of nematodes by this fungus occurred on complex hyphal structures (traps) and was effectuated by an adhesive coating, present on these trap cells. The adhesive layer was largely fibrillar in nature and was absent on cells of normal hyphae. Following capture, penetration hyphae were formed at those sites where the trap cell wall was anchored to the nematode cuticle by the adhesive. New walls of these hyphae were formed underneath the original trap cell walls, which were partly hydrolysed to allow growth and development of the penetration tubes through the adhesive coating towards the cuticle. Our observations indicated that the cuticle of the nematode was subsequently penetrated by the penetration tubes by mechanical means. After penetration a large infection bulb was formed from which trophic hyphae arose. Cytochemical experiments indicated that the sites of penetration of the cuticle were intensely stained for acid phosphatase activity. At later stages of infection activity of this enzyme was present throughout the nematode contents; the enzyme was most probably secreted by complex membranous structures associated with the cytoplasmic membrane of the infection bulb and the trophic hyphae.

INTRODUCTION

Nematode-trapping fungi comprise a group of fungi which attack living nematodes with the aid of adhesive or mechanical trapping devices (Barron, 1977). These specialized mycelial structures are remarkable since they not only firmly hold on to the nematodes but also bring about penetration of the nematode cuticle and digestion of the nematodes by trophic hyphae. In previous studies it has been shown that the fungus *Arthrobotrys oligospora* Fresenius traps nematodes in adhesive mycelial networks (Nordbring-Hertz, 1972; Nordbring-Hertz and Stålhammar-Carlemalm, 1978). These traps are hyphal structures, but differ from cells of normal vegetative hyphae in that they characteristically contain a large number of electron-dense bodies, while an adhesive coating is present on their cell wall (Nordbring-Hertz, 1984; Veenhuis et al., 1985). Electron-dense bodies have also been observed in trap cells of other nematophagous fungi and it has been suggested that these organelles are involved at some stage in the capture and/or digestion of prey (Heintz and Pramer, 1972; Dowsett et al., 1977; Nordbring-Hertz and Stålhammar-Carlemalm, 1978; Dowsett and Reid, 1979; Tzean and Estey, 1979; Wimble and Young, 1983).

Recently, evidence was obtained that the dense bodies in *A. oligospora* are peroxisomal in nature (Veenhuis et al., 1984). However, their significance in the trapping and subsequent digestion of nematodes remained speculative. To elucidate the mechanisms involved in capture and penetration of nematodes by traps of *A. oligospora* and the possible role of the dense bodies in these processes, we decided to perform time-dependent studies on nematode – fungus interactions using different ultrastructural techniques.

In this investigation we present results from an ultrastructural study on the sequence of interactions between the fungus and the nematode starting with the adhesion of the nematode to the surface of the trap, the subsequent penetration of the cuticle and the initial stages of colonization of the nematode. Special attention has been paid to the structure of the adhesive coating and its role in trapping and penetration.

MATERIALS AND METHODS

Organism and growth conditions

Arthrobotrys oligospora Fres. (ATCC 24927) was maintained on corn meal agar (CMA Difco) supplemented with K_2HPO_4 2 $g \cdot l^{-1}$. A conidial suspension from about 10-day cultures was inoculated onto dialysis membranes (Nordbring-Hertz et al., 1984) which were placed on agar plates supplemented with low-nutrient medium (LNM) and a trap-inducing peptide, phenylalanyl-valine (phe-val) (Nordbring-Hertz, 1973). Growth and trap formation on the dialysis membrane were followed by light microscopy (Nordbring-Hertz et al., 1984).

For capture experiments, the bacteria-feeding nematode *Panagrellus redivivus* Goodey, grown axenically on soya peptone – liver extract medium (Nordbring-Hertz, 1977), was used.

Fungus-nematode interaction

Interaction studies between fungus and nematodes were performed on dialysis membranes according to Nordbring-Hertz et al. (1984). A surplus of nematodes (about 100) was added to a trap-containing colony on a piece of dialysis membrane (approximately 1 cm^2) and the interaction immediately observed in the dissecting microscope. Within the first 1–2 min about five to ten nematodes were captured; subsequently the uncaptured nematodes were removed with a jet of water. The interacting organisms were fixed directly on the dialysis membrane at specific time intervals, namely 0, 10, 20, 30 min and 1, 2, 4 and 6 h after capture.

Cytochemistry

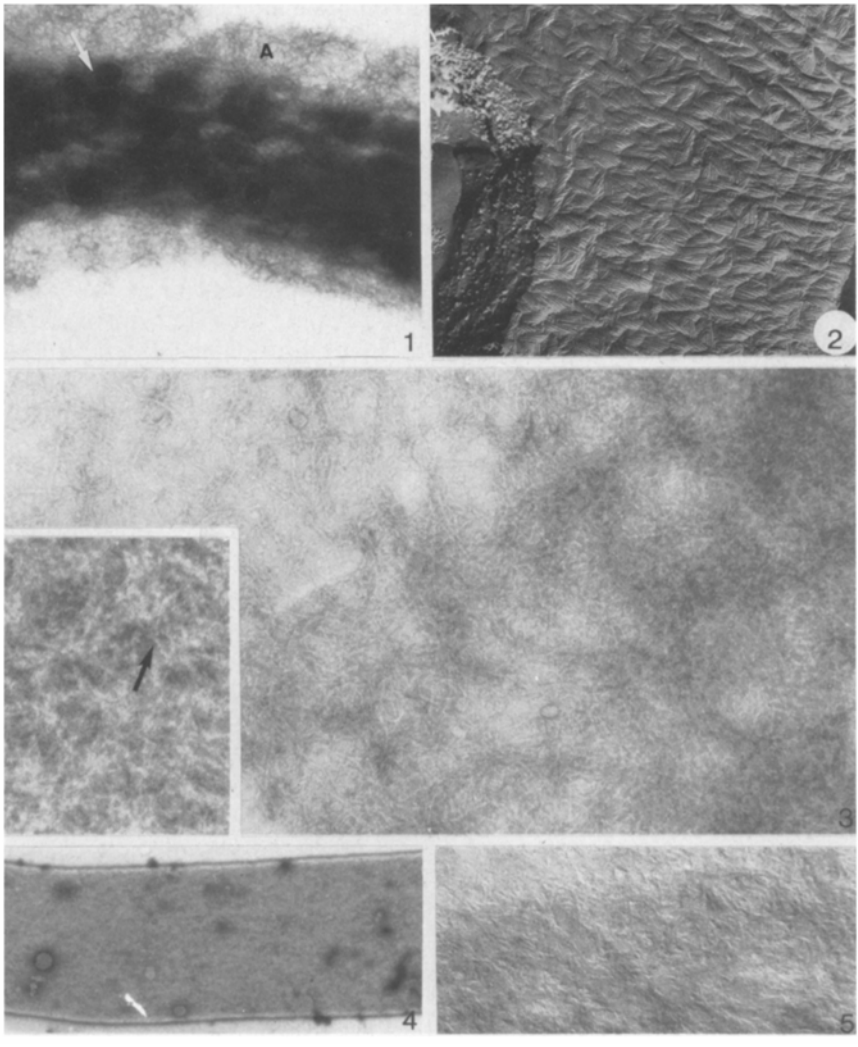
Cytochemical staining for the localization of acid phosphatase activity was performed on formaldehyde/glutaraldehyde-fixed samples (Nordbring-Hertz et al., 1984). Enzyme activity was demonstrated after incubation with Ce^{3+}-ions and the enzyme-specific substrate, β-glycerophosphate (Veenhuis et al., 1980). Controls were performed in the absence of substrate or in the presence of 10 mM NaF as an inhibitor of acid phosphatase activity.

Freeze-etching, cryosectioning, direct preparation methods

Freeze-etch replica's were prepared from traps, taken from dialysis membranes, in a Balzer's freeze-etch unit by the methods of Moor (1964). Cryosections were prepared from formaldehyde/glutaraldehyde-fixed cells on an LKB-cryokit by the methods of Tokuyasu (1978) and poststained with 1% (w/v) uranyl acetate. In addition, isolated traps were examined directly in the electron microscope after negative staining with 1% (w/v) uranyl acetate or after shadowing with platinum/carbon in a modified Edwards Vacuum Coating unit (shadowing angle 25°).

Electron microscopy

Intact cells – also after cytochemical experiments – were fixed in 1% (w/v) $KMnO_4$ in distilled water for 20 min at room temperature. In addition, cells were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 for 2 h at 0°C, followed by postfixation in 1% OsO_4 + 2.5% $K_2Cr_2O_7$ in the same buffer at room temperature. In capture experiments the material was prefixed in a mixture of 1.5% (v/v) formaldehyde + 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 2 h, washed in distilled water and postfixed in $KMnO_4$ or in $OsO_4/K_2Cr_2O_7$ as above. In some experiments the material was directly fixed in $KMnO_4$ without prefixation. After poststaining in 1% (w/v)



Figs 1–5. Substructure of the adhesive coating present on the trap cells of *Arthrobotrys oligospora*. The presence of this coating on these cells is demonstrated after cryosectioning (Fig. 1; 20000 ×); the trap cell is recognized by the dense bodies (arrow). After freeze-etching a rodlet pattern is observed (Fig. 2; 30700 ×). In direct preparations, negatively stained with uranyl acetate, the fibrillar nature of the coating (Fig. 3; 47300 ×), which is lacking on normal hyphae prepared in a similar fashion (Fig. 4; 9300 ×) is clearly observed. In the high magnification (inset Fig. 3; 120000 ×) also small globular inclusions (arrow) can be seen. The rodlet appearance of the surface is also evident after shadowing of isolated traps with platinum/carbon (Fig. 5; 17100 ×).
Note. All micrographs, presented here and elsewhere, are of cells fixed/postfixed in $KMnO_4$ unless otherwise stated. A, adhesive coating.

uranylacetate (4–8 h) the material was dehydrated in a graded alcohol series and embedded in Epon 812 as described previously (Nordbring-Hertz et al., 1984). Thin sections were cut on an LKB-Ultratome with a diamond knife and examined in a Philips EM 300 without additional staining.

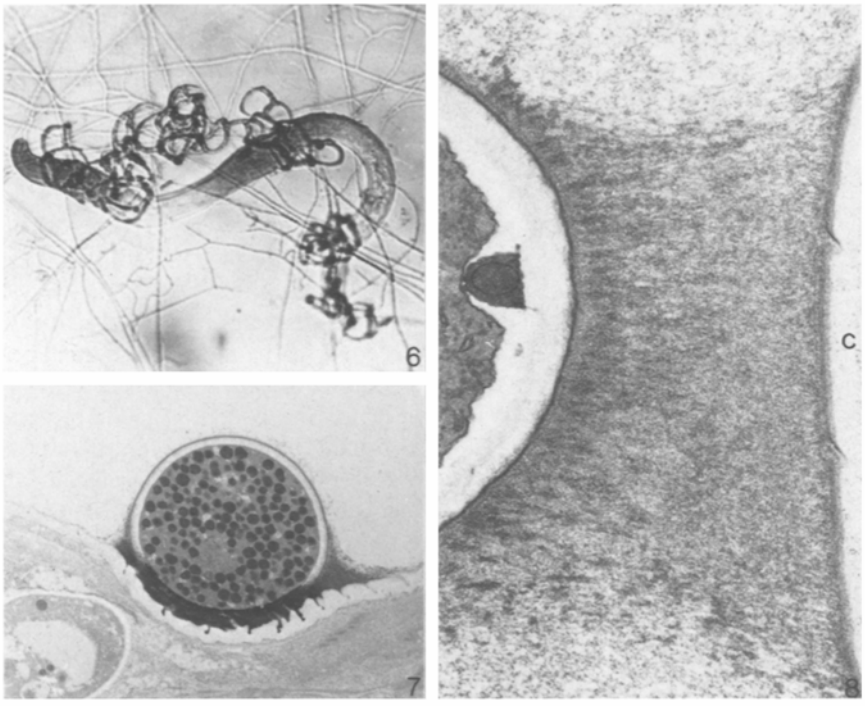
Results

Capture of nematodes

Ultrathin cryosections, prepared from mature traps indicated that the individual hyphae which constitute the trap had an adhesive coating which was fibrillar in nature (Fig. 1). Trap cells can easily be recognized from normal hyphal cells since they contain large numbers of dense bodies (Nordbring-Hertz et al., 1984; see also Fig. 18). Judged by this criterion it became evident that the presence of a fibrillar coating was confined to the trap cells and was invariably absent from normal vegetative hyphae. In freeze-etch preparations the surface of the traps showed a rodlet pattern (Fig. 2). The substructure of the adhesive was further studied using direct preparation methods. In negatively stained preparations the fibrillar nature of the coating is clearly seen (Fig. 3). At high magnification the presence of inclusions of small globules of unknown composition can be observed in the fibrillar matrix (inset Fig. 3). Also in negatively stained preparations normal vegetative cells lacked the fibrillar coating (Fig. 4). The rodlet pattern of the traps is also evident in preparations, which were directly shadowed with platinum/carbon (Fig. 5). Fig. 6 shows a light micrograph of a newly captured nematode, attached to the adhesive networks of *A. oligospora,* after post-fixation in $KMnO_4$ and embedding in Epon 812. Electron-microscopical preparations showed that the anchoring of the nematode to the trap generally had occurred at different places on the nematode's cuticle and was mediated by the fibrillar adhesive. In the early phases of trapping and during the subsequent penetration the fibrils of the adhesive now were oriented in one direction (Fig. 8). Indentations of the cuticle prior to penetration were often observed (Fig. 7).

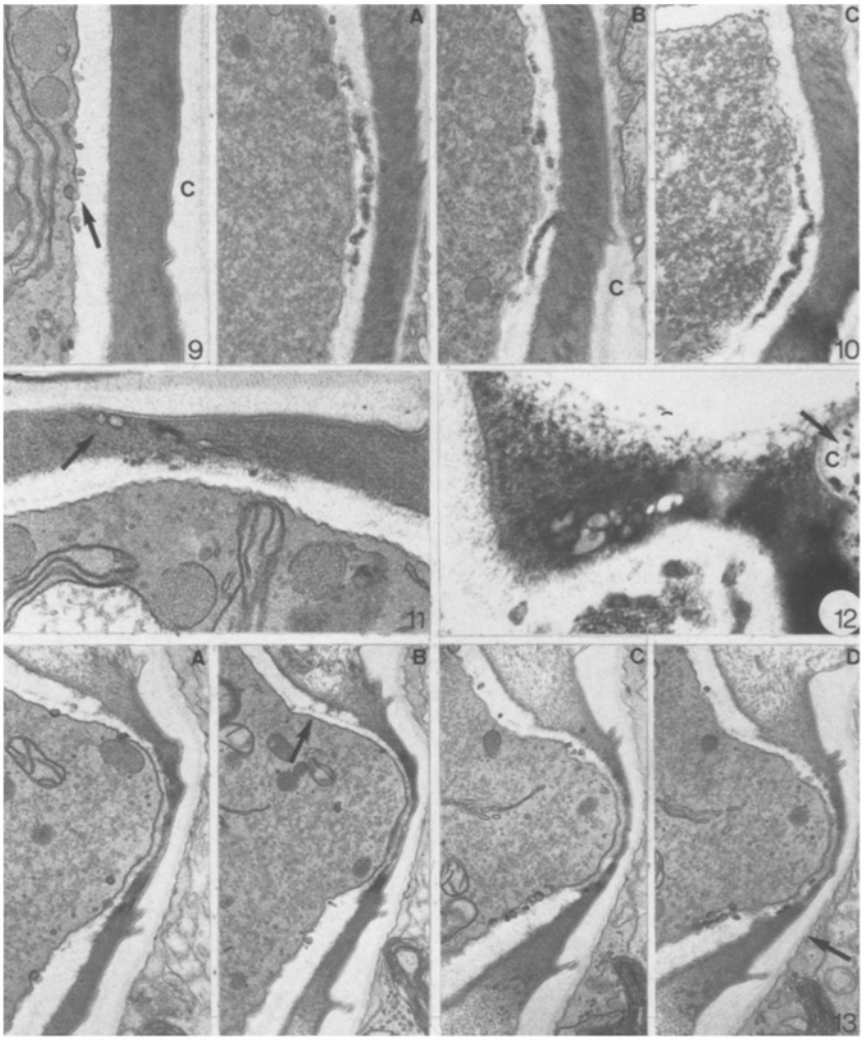
Development of the penetration tube and penetration of nematodes

Observations of thin sections of aldehyde/$KMnO_4$-fixed cells indicated that the first penetration of the nematodes by fungal hyphae generally took place within a period of 2–4 h after capture. The mechanisms involved in this process have been analysed in several series of serial sections prepared from nematodes after different times of capture. These studies revealed that the first penetration hyphae invariably developed at those places on the trap cell wall where it was effectively anchored to the nematode cuticle by the adhesive. Formation of the penetration tube was associated by the development of a number of small vesicles in localized areas close to the wall; they occurred in close association with

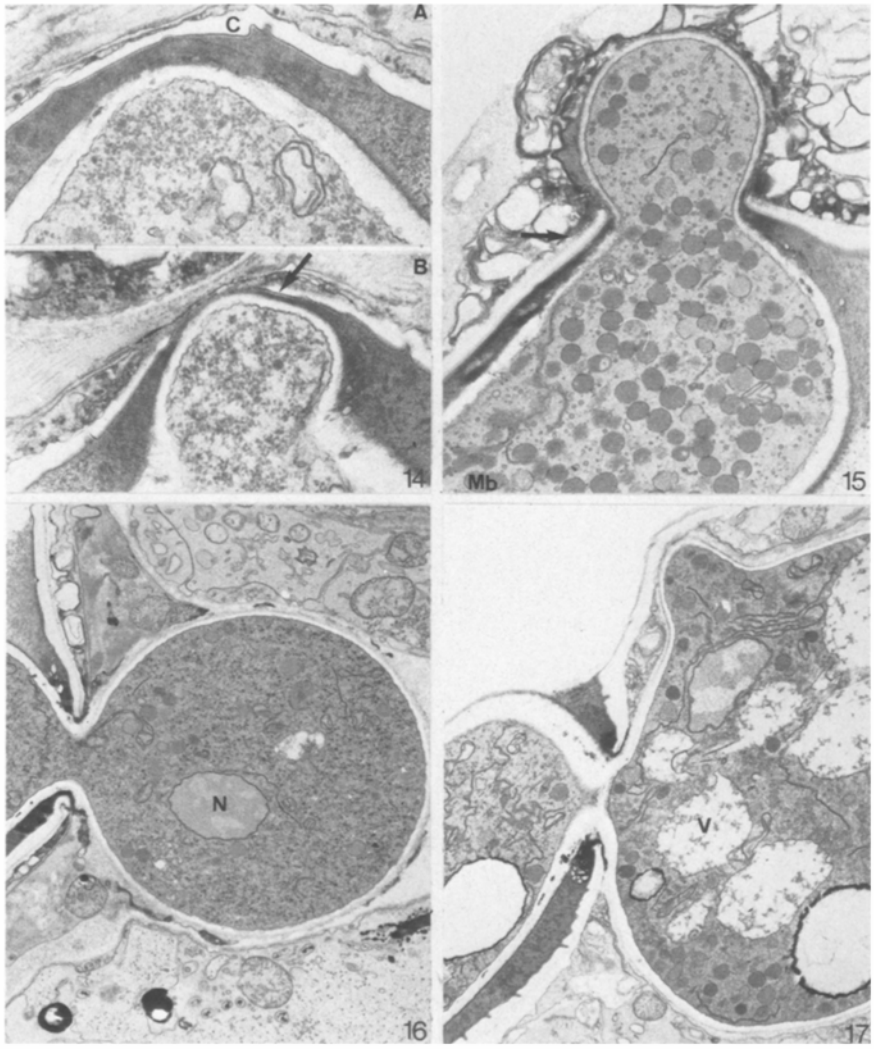


Figs 6–8. Capture of nematodes. Fig. 6 shows a light micrograph of a captured nematode after fixation in formaldehyde/glutaraldehyde-$KMnO_4$ and embedding in Epon; 2500 ×. In thin sections indentations of the cuticle were often observed (Fig. 7; 3500 ×). The fibrils of the adhesive coating are now oriented in one direction at the sites of capture (Fig. 8; 26000 ×).
See note, Figs 1–5. C, cuticle.

the plasmalemna (Fig. 9). Subsequently new cell-wall material was deposited at the cytoplasmic site of these areas with the result that the vesicles were now present between the original wall of the trap cell and this newly formed wall (Fig. 10A). Probably the vesicles, which remained unstained after staining for acid phosphatase (Fig. 12) contain enzymes involved in a local hydrolysis of the original wall of the trap cell, to allow the developing tube to come through this wall. An initial stage of this process is shown in Fig. 10A-C. These vesicles are not considered artefacts due to preparation methods since their presence was independent on whether the cells were fixed in $KMnO_4$ alone, aldehyde-$KMnO_4$ or in aldehyde-$OsO_4/K_2Cr_2O_7$ mixtures. After the new wall had come through the original cell wall of the trap, the vesicles were frequently seen randomly distributed in the adhesive coating (Fig. 11). They remained intact for prolonged periods and were still observed in samples fixed, for instance, 32 h after capture (M. Veenhuis, unpubl. results; see also Fig. 23). Subsequently, a



Figs 9–13. Development of the penetration tube. In Fig. 9 the initial stage of vesicle formation (arrow) associated with the cell membrane of the trap cell is visualized; 20000 ×. Fig. 10A-C represent three micrographs from a series of sections demonstrating the development of the wall of the penetration tube; 14700 ×. The newly synthesized wall and the site where it comes through the original wall (Fig. 10B) are indicated by arrows. In Fig. 11 the presence of vesicles in the adhesive coating is shown after the new wall (arrow) had come through the original wall of the trap cell; 31300 ×. The vesicles remained unstained after cytochemical staining for acid phosphatase activity (Fig. 12; 43300 ×; compare the positively stained parts of the cuticle, indicated by the arrows; $OsO_4/K_2Cr_2O_7$). Fig. 13A-D. Four serial sections through a developing penetration tube, prior to penetration. The cell wall of the tube arises from underneath the original wall of the trap cell (Fig. 13B; arrow). Note the indentation and decrease in thickness of the nematode cuticle, which is virtually intact since the dense outer layer and the fibrous inner layer (see Fig. 13D; arrow) at the inside still can be observed (Fig. 13A-D; 14000 ×).
See note, Figs 1–5. C, cuticle.



Figs 14–17. Initial stages of penetration. Fig. 14A-B (19500 ×) represent two micrographs of a series of sections through a penetration hypha which is just penetrating the cuticle (Fig. 14B). As is shown in this picture, and also in Fig. 14A, the cuticle is not hydrolysed prior to penetration since the dense outer layer of the cuticle still can be seen (arrow). In Fig. 15 the initial stage of an infection bulb is shown. Note the presence of electron-dense microbodies in the developing bulb; 12700 ×. A fully developed infection bulb is shown in Fig. 16; 13300 ×. Fig. 17 represents an initial stage of the formation of a trophic hypha. In addition, a cross wall is generally formed in the neck between the original trap cell and the infection bulb; 9300 ×. Note the presence of vesicles in the adhesive coating in Figs 16 and 17.
See note, Figs 1–5. C, cuticle; Mb, electron-dense microbody; N, nucleus; V, vacuole.

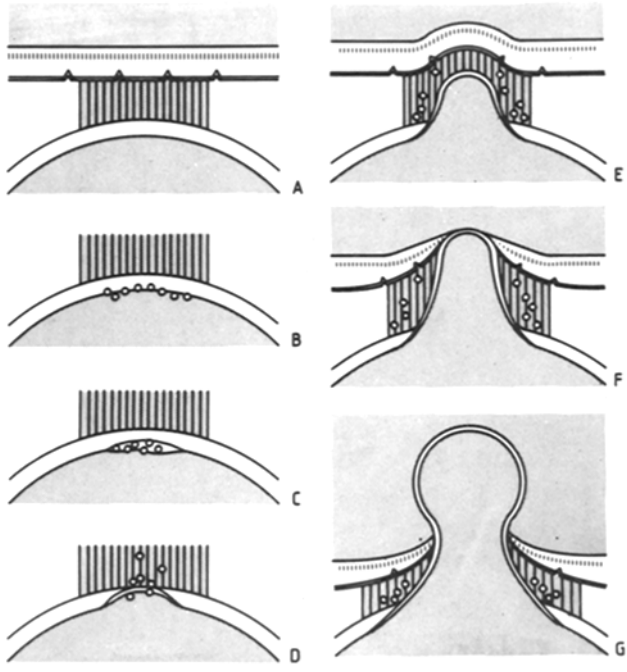
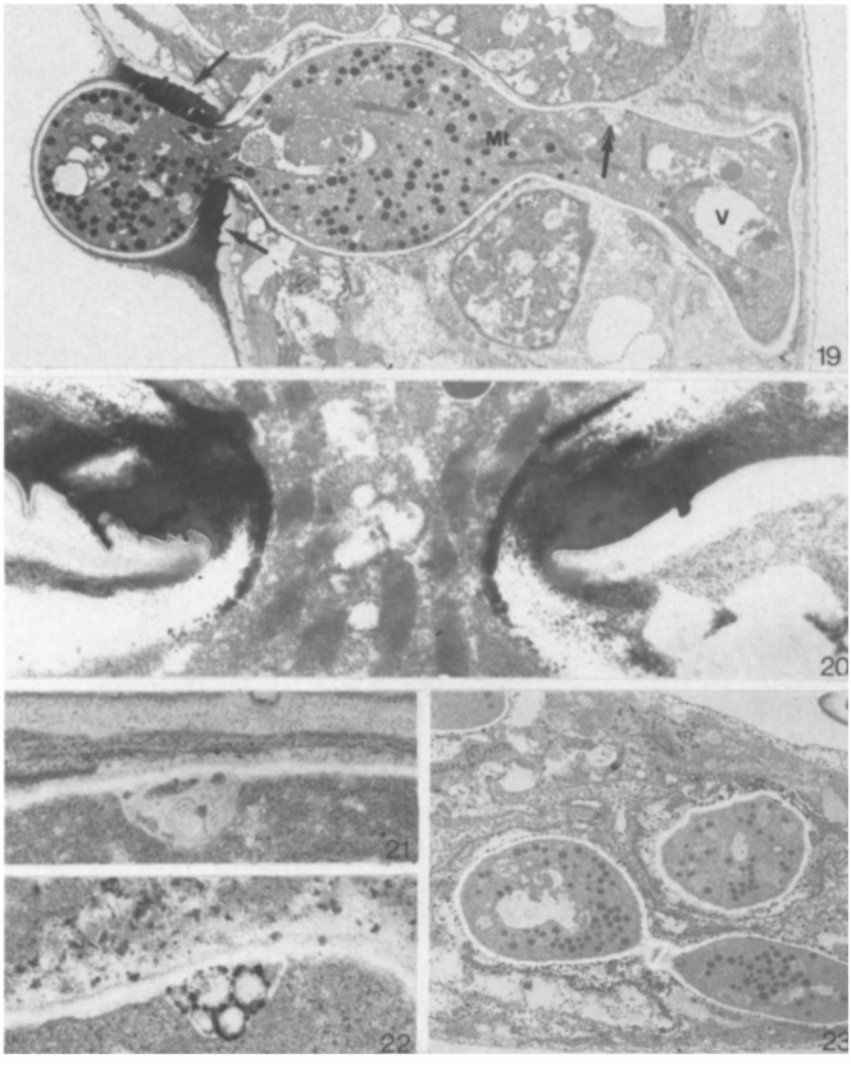


Fig. 18. Schematic drawing of the subcellular events occurring during penetration of a nematode. Capture (Fig. A) is followed by the formation of small vesicles in association with the cell membrane of the trap cell (Fig. B) and the deposition of new cell-wall material (Fig. C). After partial hydrolysis of the original trap cell wall (Fig. D) a penetration tube develops (Fig. E). The small vesicles are now also seen randomly distributed in the adhesive. During subsequent development of the penetration tube the nematode cuticle first becomes indentated (Fig. F) and subsequently is penetrated (Fig. G).

small penetration tube with a relatively thin cell wall developed; this tube proceeded through the adhesive coating towards the nematode cuticle. Where this tube approached the nematode, invariably indentations of the cuticle were observed, most probably as a result of the mechanical force brought about by the growing penetration tube (Fig. 13A-C).

The following observations suggest that the nematode cuticle is finally penetrated, at least largely, by mechanical force: (i) the observed decrease in thickness of the cuticle at this stage is most probably not caused by enzymatic action. In thin sections the dense outer layer and the fibrous inner layer at the inside were still intact (compare Figs 13 and 14A-B), indicating that no hydrolysis of the cuticle had occurred; (ii) thinning of the cuticle at the site of the penetration tube is associated with a decrease in depth of the periodical indentations that are originally present in the cuticle (Figs 13 and 14), suggesting localized stretching of the cuticle. This stretching of the cuticle is furthermore indicated by an



Figs 19–23. Cytochemical staining of acid phosphatase at different stages of penetration. Four h after capture staining is confined to the site of penetration (arrows, Fig. 19; 8600 ×). High magnification revealed that the reaction products are localized at the cell membrane, the cell wall and part of the adhesive (Fig. 20; 40700 ×). At this stage the membranous structures which developed in the trophic hyphae are unstained (Fig. 21; 32000 ×. See also Fig. 19, double arrow). These structures are heavily stained for acid phosphatase in samples 6 h after capture (Fig. 22; 32000 ×). In addition, also the nematode contents, including the cuticle, are now positively stained for acid phosphatase (Fig. 23; 4600 ×).
See note, Figs 1–5. Mt, mitochondrion; V, vacuole.

increase in distance of the periodicity observed in the fibrous inner layer of the cuticle (Figs 15 and 18).

Following penetration, the infection tube is rounded (Fig. 15) and subsequently swells into a large infection bulb (Nordbring-Hertz et al., 1984; compare also Fig. 19). From these infection bulbs several trophic hyphae may develop. In contrast to earlier findings (Nordbring-Hertz and Stålhammar-Carlemalm, 1978; Dowsett and Reid, 1979; Wimble and Young, 1983) the dense bodies, typical for trap cells, also occurred in the infecting hyphae, both in the infection bulb and in the trophic hyphae (Figs 15–17, 19). A schematic drawing of the subcellular events occurring during penetration of the nematodes is shown in Fig. 18.

Cytochemical staining experiments indicated that the site of penetration is intensely stained for acid phosphatase (Fig. 19). This staining is confined to the cell membrane, cell wall and part of the adhesive coating (Fig. 20). The numerous electron-dense bodies in the cells remain unstained. Upon invasion of the nematode, specialized membranous structures are formed at the cytoplasmic membrane of the infection bulb and the trophic hyphae (Fig. 21) which at later stages of infection – 6 h after capture – were heavily stained for acid phosphatase activity (Fig. 22). At this stage also the nematodes which now contained several trophic hyphae and which were originally unstained, showed a staining for acid phosphatase throughout their cytoplasm (Fig. 23).

Discussion

The present investigation has focused on three major aspects of the capture and penetration of nematodes by adhesive network traps of *A. oligospora*: (i) the role of the adhesive coating; (ii) the role of hydrolytic enzymes and their association with specific organelles and (iii) the role of mechanical pressure and/or hydrolytic activity in the penetration process.

Previously, it has been suggested that the adhesive coating of nematode-trapping structures covers the traps immediately after their formation (Nordbring-Hertz, 1972). Also an increased secretion of mucilage in the presence of prey has been demonstrated (Nordbring-Hertz and Stålhammar-Carlemalm, 1978; Dowsett and Reid, 1979). In the present study we demonstrated that the adhesive coating is largely fibrillar in nature and of considerable thickness (Figs 1–5). A change in the arrangement of the fibrils of the adhesive material is seen at the site of capture of nematodes before penetration (Fig. 8). At the penetration spot fibrils directed more or less perpendicularly to the cuticle are present in the immediate vicinity of the penetration hypha. After freeze-fracturing the adhesive material covering the trap cells showed a rodlet pattern; this configuration is comparable to the interwoven appearance of the surface structure of different fungal conidia (Ghiorse and Edwards, 1973; Hashimoto et al., 1976; Cole et

al., 1979). The presence of the rodlet pattern on a hyphal structure such as the adhesive trap (but not on normal hyphae) is interesting, since trap and conidiophore-conidial formation is interconnected in several species (Jansson and Nordbring-Hertz, 1981). Our results indicate that the rodlet layer indeed constitutes the surface of the adhesive layer of the trap.

Acid phosphatase activity was detected in the adhesive, in the cell wall and cell membrane at the site of penetration. After penetration, activity of the enzyme was furthermore observed in specific membranous structures associated with the cell membrane of the infection bulbs and the trophic hyphae (Fig. 22) suggesting that the fungus actively degrades its prey. The increased staining of the entire nematode for acid phosphatase within a 6-h period is in agreement with previous light-microscopic observations (Toth et al., 1980). In trap cells that have not been in contact with the nematode, hydrolytic activity was confined to the vacuoles (Nordbring-Hertz et al., 1984; Veenhuis et al., 1984). On the other hand, the numerous dense bodies present in the trap cells were not stained for hydrolytic enzymes. In the present study evidence for their involvement in either adhesion or penetration, as was suggested previously for different nematophagous fungi (Heintz and Pramer, 1972; Dowsett et al., 1977; Nordbring-Hertz and Stålhammar-Carlemalm, 1978; Tzean and Estey, 1979), was not obtained. A possible role of the dense bodies in the digestion of the nematode is equally unclear (Veenhuis et al., 1985).

In a previous study including capturing on an agar surface (Nordbring-Hertz and Stålhammar-Carlemalm, 1978), it was shown that penetration of the nematode cuticle took place within 1 h after capture. In the present study we noted differences between individual nematodes, but generally the sequence of events which led to the penetration of the nematode was somewhat slower on the dialysis membrane. The indentations, observed at the spots of penetrations and the absence of visible signs of hydrolysis of the cuticle, suggest that mechanical force is involved in the penetration process. A prerequisite for the fungus to penetrate the nematode against the osmotic pressure of the intact animal is a firm anchoring of the trap cell to the cuticle by the adhesive. That this is indeed the case may be concluded form the observations that the connection between the trap cell and the cuticle is not disrupted during penetration while in addition the distance between the cuticle and the wall of the penetrating trap cell from which the penetration tube arose remained virtually unaltered (Figs 9–15). However, the presence of hydrolytic enzymes (as exemplified by acid phosphatase) in the adhesive at the site of penetration may indicate that the cuticle is weakened at this point. On the other hand, this phenomenon may also indicate an initial secretion of hydrolytic activity which occurs simultaneously with the first penetration. This is strengthened by the finding that at later stages of penetration acid phosphatase, most probably synthesized by the infection hyphae, is now distributed over the whole nematode contents.

The small vesicles, present in the adhesive in the vicinity of the penetration

tube, were not stained for acid phosphatase activity. They are most probably solely involved in – partial – lysis of the cell wall of the trap. This is necessary to allow development of the penetration hypha, which is initially formed underneath the original cell wall of the trap, like described for budding of ascomycetous yeasts (Kreger-Van Rij and Veenhuis, 1972). The formation of an infection bulb (Shepherd, 1955) is a further indication of the high osmotic pressure of the intact nematode and has the further advantage that the site of penetration is efficiently sealed.

In the present study it became evident that in traps, which had captured/penetrated nematodes also individual trap cells may be penetrated by each other. The significance of this – unexpected – observation is under current investigation.

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